Food Control 27 (2012) 146–152

Contents lists available at SciVerse ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Production of the monoclonal antibody against Sudan 4 for multi-immunoassay of Sudan dyes in egg

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ARTICLE INFO

Article history:
Received 15 January 2012
Received in revised form
19 March 2012
Accepted 19 March 2012

Keywords:
Sudan dyes
Sudan 4
Monoclonal antibody
Heterologous immunoassay
Egg

ABSTRACT

Sudan dyes (Sudan 1, 2, 3, 4, Para red and Sudan red G) have been banned as food colorants due to their carcinogenicity. This study first synthesized a hapten of Sudan 4 and produced the monoclonal antibody for immunoassay of these red dyes. The hapten was synthesized by coupling 4-amino-3-methylbenzoic acid to *o*-toluidine to obtain an intermediate hapten and then the intermediate hapten was coupled to β-naphthol to obtain a tentative hapten. The generated monoclonal antibody showed broad specificity to the six Sudan dyes. After evaluation of different coating antigens, a heterologous competitive indirect immunoassay was developed to determine the six red dyes in egg yolk simultaneously. The cross-reactivities for the six analytes were in a range of 64%–110% and the limits of detection were in a range of 0.08–0.2 ng/g depending on the compound. Intra- and inter-assay recoveries from the standards fortified blank samples ranged from 71.5% to 107.4% with coefficients of variation lower than 14.6%.

1. Introduction

Sudan dyes are a class of synthetic azo dyes that are usually used in industry or printing. Due to its potential carcinogenicity to animals in laboratory experiments (Ahlstrom, Sparr Eskilsson, & Bjorklun, 2005; Pinheiro, Touraud, & Thomas, 2004; Stiborova, Martinek, Rydlova, Hodek, & Frei, 2002), Sudan 1 (S1) has been classified as a category 3 carcinogen by International Agency for Research on Cancer (IARC, 1975: pp. 224–231). Another Sudan dye Para red (PR) was also assumed a genotoxic carcinogen by the independent scientific experts of UK Food Standards Agency (UK Food Standards Agency, 2008). Recently, Xu and the coworkers have proven S1, Sudan 2 (S2), Sudan 3 (S3), Sudan 4 (S4) and PR can be reduced to the carcinogenic aromatic amines by some prevalent species of human intestinal bacteria (Xu, Heinze, Chen, Cerniglia, & Chen, 2007; Xu, Heinze, Paine, Cerniglia, & Chen, 2009). Sudan red G (SG) is also an azo dye besides the five red dyes. The chemical structures of the six dyes are shown in Fig. 1. In consideration of their potential harm effects, many countries have forbidden the use of them as food colorants (Commission decision of EC, 2003). However, these red dyes may be illegally added into chilli-, curry- or palm-oil as colorants and S1 was even found in China in meat and eggs (He et al., 2007). Therefore, it is important to inspect the presence of these red dyes in foods.

Recent years, HPLC (Ertas, Ozer, & Alasalyar, 2007; Li, Yang, Zhang, & Wu, 2009; Wu, Yang, Zhao, HuangFu, & Shen, 2009), GC/MS (He et al., 2007), and LC-MS/MS (Hou, Li, Cao, Zhang, & Wu, 2010; Mazzotti et al., 2008; Sun, Wang, & Ai, 2007) have been usually used to determine the residue of these red dyes in various foods. These methods can qualitatively and quantitatively determine these dyes, but they are time-consuming, and sophisticated extraction procedures and expensive instruments are required compared to enzyme linked immunosorbent assay (ELISA). ELISA is a low cost and sensitive method capable of screening large amount of samples in a single test.

There have been some researchers developing ELISA method for the determination of Sudan dyes in various foodstuffs (Anfossi, Baggiani, Giovannoli, & Giraudi, 2009; Chang et al., 2011; Han et al., 2007; Ju, Tang, Fan, & Chen, 2008; Oplatowska, Stevenson, Schulz, & Elliott, 2011; Wang et al., 2009; Xu, Wei et al., 2010; Xu, Zhang et al., 2010). However, most of the authors produced the antibodies against S1 and the obtained antibodies could only detect one (Han et al., 2007; Oplatowska et al., 2011), two (Xu, Wei et al., 2010; Xu, Zhang et al., 2010), three (Ju et al., 2008; Wang et al., 2009) or at most four of the six analytes (Anfossi et al., 2009). The authors of the present paper have produced the antibody against PR for simultaneous determination of the six red dyes

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doi:10.1016/j.foodcont.2012.03.017

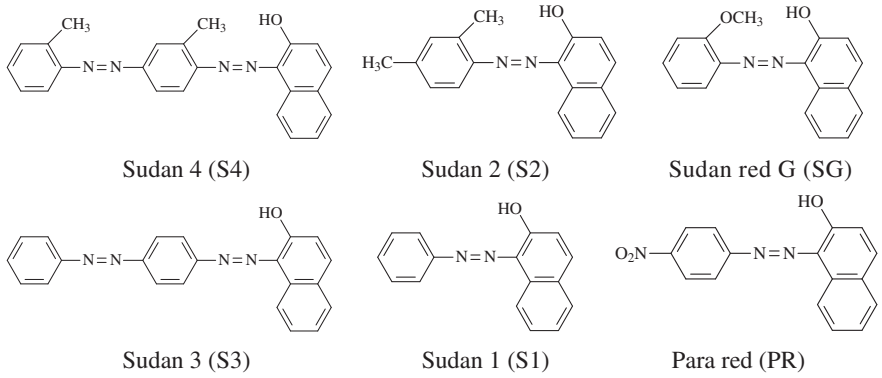

Fig. 1. Chemical structures of the six common Sudan dyes. The authors of this figure are Wen Chong Shan, Jian Zhong Xi, Jie Sun, Yu Jie Zhang, Jian Ping Wang.

(Chang et al., 2011). The antibodies described above all showed high cross reactivity to S1, S3 and PR, but showed low or negligible cross reactivity to S2, S4 and SG. Recently, we synthesized a hapten of S2 by covalent coupling 4-amino-3-methylbenzoic acid to β-naphthol to produce the monoclonal antibody (Liu, Zhang, Zhang, Gao, & Wang, 2012). The obtained antibody showed broad specificity to the six red dyes with high and comparable cross-reactivities (CR).

As shown in Fig. 1, the molecules of the six red dyes all contain a same core structure, 1-phenylazo-2-naphthalenol. The previous antibodies against S1 and PR whose molecules only contain the core structure did not give the satisfactory results (Anfossi et al., 2009; Chang et al., 2011; Han et al., 2007; Ju et al., 2008; Oplatowska et al., 2011; Wang et al., 2009; Xu, Wei et al., 2010; Xu, Zhang et al., 2010). Our recent study has shown the antibody of S2 simultaneously recognized the six Sudan dyes due to its more complex molecule than that of S1 and PR (Liu et al., 2012). Among the six red dyes, S4 contains the bulkiest and the most complex molecule and the molecules of other five compounds can all be regarded as a part of S4 (Fig. 1). Then, we speculated the antibody of S4 should be the broad specific antibody for these Sudan dyes. In this study, Sudan 4 was used to produce the class generic monoclonal antibody and develop an immunoassay for simultaneous determination of the six red dyes in egg.

2. Experimental

2.1. Materials

Sudan 1 (S1), Sudan 2 (S2), Sudan 3 (S3), Sudan 4 (S4), Para red (PR), bovine serum albumin (BSA), ovalbumin (OA) and Freund's adjuvants were purchased from Sigma–Aldrich (Shanghai, China). Sudan red G (SG) was obtained from Shanghai Jingchun reagent company (Shanghai, China). 3, 3′, 5, 5′- tetramethylbenzidine (TMB) was purchased from Serva (Heidelberg, Germany). 4-amino-3-methylbenzoic acid, *o*-toluidine, β-naphthol and other chemical reagents were all analytical grade or better from Beijing chemical company (Beijing, China). Microtiter plates were supplied by Greiner Bio-one (Germany).

Stock solutions of each compound (100 μg/mL) were prepared with acetonitrile and stored at −20 °C to be stable for 3 months. Working solutions with series concentrations (0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10, 20, 50 ng/mL) were diluted from stock solutions with PBS. All the working solutions were stored at 4 °C to be stable for at least 4 weeks. PBS (pH 7.2) was prepared by dissolving 0.2 g KH_2PO_4, 0.2 g KCl, 1.15 g Na_2HPO4, and 8.0 g NaCl in 1000 mL demineralised water. Washing buffer (PBST) was PBS buffer containing 0.05% Tween. Coating buffer was carbonate buffer (0.1 M, pH 9.6). Substrate buffer was 0.1 M citrate (pH 5.5). The substrate system was prepared by adding 200 μL TMB in DMSO (1%, w/v) and 64 μL 0.75% (w/v) H_2O_2 into 20 mL substrate buffer.

2.2. Synthesis of the hapten S4D

The synthesis of the hapten was divided into two steps and the synthetic processes are shown in Fig. 2. The first step was to couple 4-amino-3-methylbenzoic acid to *o*-toluidine by using of diazotization method. About 151 mg (1 mmol) 4-amino-3-methylbenzoic acid was dissolved in 5 mL of 2 M hydrochloric acid under stirring and the solution was cooled down to 4 °C. Then a solution of 0.1 M $NaNO_2$ was continually added into the above solution dropwisely. During this process, the reaction procedure was monitored by using potassium iodide-starch test paper until the test paper turned blue (solution 1). About 107 mg (1 mmol) *o*-toluidine was dissolved in 2 mL of sodium bicarbonate solution and the solution was cooled down to 4 °C (solution 2). Solution 2 was then added dropwise into solution 1 under gentle stirring. The mixture was stirred for 30 min at 4 °C to obtain some red sediment. Then the pH of the solution was adjusted to 3.0 with concentrated hydrochloric acid prior to another 30 min stirring. Finally, the mixture was filtered under vacuum and the obtained red residue was washed with 50 mL water, and subsequently dried to yield the intermediate hapten.

The second step was to couple the intermediate hapten to β-naphthol by using of diazotization method (Fig. 2). The coupling procedure was carried out as described above just displacing 4-amino-3-methylbenzoic acid with 120 mg (0.5 mmol) intermediate hapten and displacing *o*-toluidine with 70 mg (0.5 mmol) β-naphthol. Finally, the tentative hapten S4D was obtained (IR (KBr) V_{max} 3567, 3218-2320, 3075, 2973, 1683, 1508, 1251, 1203, 842, 742, 669 cm^{-1}).

2.3. Preparation of the conjugates

The hapten S4D was coupled to carrier protein by using of mixed anhydride method. The preparation process is shown in Fig. 2. About 3 mL of *N*, *N*-dimethylformamide dissolving 21 mg hapten and 30 μL triethylamine were added into a glass jar. Then 30 μL of isobutyl chloroformate was added and the mixture was stirred for 60 min at 4 °C. And then, the obtained solution was added dropwise into 3 mL of PBS containing 74 mg BSA or 40 mg OA and stirred for 12 h at 4 °C. The obtained immunogen (S4D-BSA) and coating antigen (S4D-OA) were dialyzed against three changes of PBS for 3 days and stored at −20 °C until used. The conjugates, carrier protein, S4 and S4D were scanned on a UV–Vis spectrophotometer to identify the conjugation, and the hapten/protein coupling ratios